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L-cysteine is a potent inhibitor of protein glycation on both albumin and LDL, and prevents the diabetic complications in diabetic–atherosclerotic rat

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ABSTRACT

The aim of the present study is to investigate the protective effect of L-cysteine (Cys) on protein glycation, and hence, the improvement of diabetic complications. Streptozotocin-induced diabetic rats on an atherogenic diet were used as a model. Two groups of rats, normal and diabetic, were treated with 0.05% of Cys in drinking water for 3 months and two others received water only. Some parameters including: glucose, insulin, glycation products, activity of the glyoxalase system, lipid profile, oxidation markers, high sensitivity CRP (Hs CRP) and creatinine in the serum, and protein in the urine of all rats were determined. Furthermore, rat serum albumin and LDL were purified and incubated with glucose in the presence and absence of Cys and the samples were investigated for glycation and oxidation products. Cys showed an inhibitory effect on glycation and oxidation products in both in vivo and in vitro conditions. It reduced glucose, insulin resistance, triglyceride, cholesterol, LDL, Hs CRP and creatinine in the serum; and the proteinuria; furthermore, it increased HDL and glyoxalase system activity in the serum of the diabetic–atherosclerotic rats. Cys reduced all of the risk factors of diabetic complications and showed anti-atherosclerotic potential; thus it is useful in diabetes treatment.

Introduction

Hyperglycemia induces ROS[1] overproduction (Du, Miller, & Kern, 2003). Excess formation of ROS in addition to the disorder in the status of the antioxidant defense system, plays a critical role in *diabetes*-related *vascular complications*. GSH[2] is involved in the antioxidant defense system and its deficiency contributes to pathogenesis of diabetes (Wu, Fang, Yang, Lupton, & Turner, 2004). Cys[3] availability has been considered as a major rate limiting factor in glutathione production (Griffith, 1999). It has been shown that the levels of both Cys and GSH decreased in diabetes (Sekhar et al., 2011).

In addition, hyperglycemia induces glycation of biomacromolecules and the formation of AGEs.[4] All of the early, intermediates and final AGEs are involved in the initiation and development of vascular diabetes complications (Brownlee, 1995; Cohen, 2003). Elevated levels of the g-Alb[5] are associated with the metabolic disorders observed in diabetes mellitus (Brownlee, 1995; Cohen, 2003). It has been reported that inhibiting excess non-enzymatic glycation of serum albumin improves renal abnormalities and protects against the development of renal insufficiency (Cohen et al., 2000).

Carbonyl stress is a phenomenon responsible for activation of a series of inflammatory responses leading to accelerated vascular damage in diabetes (Baynes & Thorpe, 1999). Endogenous products of α-oxoaldehydes, such as GO[6] and MG[7] , are the key factors involved in this phenomena.

Elevated levels of circulating AGEs are associated with diabetics, particularly those with renal insufficiency, because these products are normally excreted in the urine (Muri, 1954). AGEs are nephrotoxic both in vitro and in vivo (Vlassara et al., 1994; Yamagishi et al., 2003). It has been shown that pharmacological intervention in AGE formation protects against structural lesions, proteinuria and renal function loss in experimental diabetes (Alderson et al., 2004).

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[1] Reactive oxygen species.
[2] Glutathione.
[3] L-Cysteine.
[4] Advanced glycation end products.
[5] Glycated albumin.
[6] Glyoxal.
[7] Methylglyoxal.

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Defects in insulin action and hyperglycemia could lead to changes in plasma lipoproteins in patients with diabetes. In poorly controlled type1 diabetes, in addition to ketoacidosis and hypertriglyceridemia, increased Chol[8] and LDL[9] and reduced HDL[10] commonly occur. An increase in dietary Chol favors the development of glomerulosclerosis (French, Yamanaka, & Ostwald, 1967; Wellmann & Volk, 1971). Lowering the serum Chol of the diabetic rat has improved the renal function and significantly decreased the formation of AGEs (Metz, Alderson, Thorpe, & Baynes, 2003).

PU[11] is an important marker for DN[12] (Abbate, Zoja, & Remuzzi, 2006) that potentially used as a surrogate marker for advanced atherosclerosis (Marso et al., 1999), CKD[13] and CVD.[14] It can also be used for monitoring the *therapeutic efficacy* (Mitch, Shahinfar, Dickson, de zeeuw, & Zhang, 2004).

The beneficial effect of L-lysine (Jafarnejad, Bathaie, Nakhjavani, Hassan, & Banasadegh, 2008) and L-glycine (Bahmani, Bathaie, Aldavood, & Ghahghaei, 2012), as the chemical chaperones of amino acid family, on diabetes complication has been recently studied in our Lab. Here, we investigated the effect of Cys in the early to end products of albumin glycation, as a model protein. The effects of three month Cys therapy on the glycation and oxidation of LDL were also studied in vitro. All of these parameters were also determined in vivo. In addition, lipid profile, insulin resistance, markers of oxidation, inflammation, and kidney function, as well as the atheromatous plaque formation were investigated in diabetic rat on atherogenic diet.

Materials and methods

Materials

Dihydroxyacetone, sodium carbonate, sodium mono and dihydrogen phosphate, Iodoacetamide,[15] Glc,[16] sodium azide, acetonitrile, ethanol, trichloroacetic acid,[17] 2,4-dinitrophenylhydrazine, potassium iodide, cyclohexane, heptafluorobutyric acid, CuSo4, thiobarbituric acid, Chol, cholic acid, Cys (L-cysteine hydrochloride monohydrate), 2-methylpropanol, CaCl2, NaCl and EDTA from Merck Chemical Co. STZ,[18] GO, MG, NBT,[19] oxalic acid, 5-hydroxymethylfurfuraldehyde,[20] and Triton X-100, were purchased from Sigma Chemical Co. The 0.45 μm syringe filters were obtained from Millipore. Standard of PEN[21] was gifted by Professor Dr. V. M. Monnier.

Animal study

Induction of diabetes and atherosclerosis, and treatment of rats with Cys

Male Wistar rats, 8 weeks old and weighing 180 ± 15 g, were purchased from the Pasteur Institute of Iran, Karaj. Animals were housed under controlled temperature with a 12 hour light and dark cycle with free access to food and water. Two weeks later they were randomly divided into four groups, and after overnight fasting, two groups received a single injection (i.p.[22]) of STZ (45 mg/kg body weight in Na-citrate buffer, pH 4.5) (Méndez & Ramos, 1994). After three days rats with FBS[23] >11 mM were accepted as diabetic. Two other control groups were injected with a vehicle only.

Thus, the experiment was carried out on four groups of rats (#10 rats in each group) that were named as follows: normal rats (N), normal rats treated with Cys (NT), diabetic rats (D), and diabetic rats treated with Cys (DT). They were fed either a standard chow diet, N and NT groups, or an atherogenic diet (Chow diet containing 1% cholesterol and 0.5% cholic acid), D and DT groups. The treatment of NT and DT groups with Cys (0.05% in drinking water) began on the first week of diabetes induction. It was chosen according to the literature (Sagara et al., 1994) and our personal experience. Based on the solubility of Cys in water (50 mg/ml, Sigma-Aldrich Product Information) and preventing the stress induced by gavage (Brown, Dinger, & Levine, 2000), this amino acid was added in drinking water; similar to our previous studies (Bahmani et al., 2012; Jafarnejad et al., 2008). The study continued up to the end of three months. A 24-hour urine specimen was collected at the end of the third month, by transferring and keeping rats in individual metabolic cage for 24 h. Then, after 16 h of fasting, rats were anesthetized with an i.p. injection of ketamine–xylosine (90 + 10 mg/kg body mass) and their blood was collected from the heart and transferred into test tubes with and without EDTA. Serum samples were prepared by 15 min centrifugation of blood at 5000 ×g and were stored at −70 °C until testing.

The experimental protocol was approved by the animal Ethical Committee in accordance with the guidelines for the care and use of laboratory animals prepared by Tarbiat Modares University.

Determination of biochemical parameters

FBS, TG,[24] Chol and HDL were measured by enzymatic colorimetric methods; LDL was calculated from the Friedwald equation (Jafarnejad et al., 2008). Cr[25] was measured in the serum of rats using the Jaffe kinetic method (Pars Azmune, Tehran, Iran).

The serum insulin level was determined by ELISA[26] using a rat insulin kit (Mercodia, Uppsala, Sweden). Then the HOMA-IR[27] and pancreas cell function were calculated using Eq. (1):

$$\text{HOMA-IR} = [(\text{fasting insulin (U/ml)} \times \text{fasting glucose [mM]})/22.5]. \quad (1)$$

Determination of AOPP[28] in the serum

AOPP was determined according to the method of Witko-Sarsat et al. (1996). Briefly, 200 μl of serum diluted 1:5 with PBS,[29] 200 μl of chloramine T (0–100 μM) for calibration and 200 μl of PBS as blank were applied. Then, 10 μl of potassium iodide (1160 mM) and 20 μl of glacial acetic acid were added and the absorbance of the solution was immediately measured at 340 nm, using a Shimadzu Spectrophotometer Model UV-3100 (Tokyo, Japan). Concentration of AOPP was expressed in chloramine units (μM).

Determination of glyoxalase system

The activity of glyoxalase system including GLO-I[30] and GLO-II[31] was measured in hemolysate. The activity of GLO-I was assayed at 37 °C by measuring the initial rate of formation of S-D-lactoylglutathione. The assay mixture contained a final volume of 1 ml, 100 mM sodium phosphate buffer, pH 7.2; 3.5 mM of MG; 1.7 of mM GSH and 16 mM of $MgSO_4$. The mixture was incubated for 10 min followed by the addition of 20 μl of hemolysate and the absorption at 240 nm was read after 2 min.

The activity of GLO-II was assayed by measuring the initial rate of hydrolysis of S-D-lactoglutathione to GSH and D-lactic acid. The initial

[8] Cholesterol.
[9] Low density lipoprotein.
[10] High density lipoprotein.
[11] Proteinuria.
[12] Diabetic nephropathy.
[13] Chronic kidney disease.
[14] Cardiovascular disease.
[15] IAM.
[16] D-glucose.
[17] TCA.
[18] Streptozotocin.
[19] Nitroblue tetrazolium chloride.
[20] 5-HMF.
[21] Pentosidine.
[22] Intraperitoneally.
[23] Fasting blood sugar.
[24] Triglyceride.
[25] Creatinine.
[26] Enzyme-linked immunosorbent assay.
[27] Homeostasis model assessment of insulin resistance.
[28] Advanced oxidation protein products.
[29] Phosphate buffered saline.
[30] Glyoxalase I.
[31] Glyoxalase II.

concentration of S-D-lactoglutathione was 0.3 mM in 50 mM Tris/HCl, pH 7.4; and the rate of its hydrolysis (after adding hemolysate) was followed by measuring the absorbance at 240 nm after 2 min. The activities of GLO-I and GLO-II were expressed as unit/ml (U/ml) (Sharma & Kale, 1993).

Determination of glycated products in the serum of rats

The FA[32] (glycated proteins in the serum) and g-Alb were quantified by a colorimetric method using NBT (Xu et al., 2002). Briefly, 50 μl of the sample was incubated with 100 μl of IAM (5 mM) for 30 min at 37 °C. Then, 1000 μl of NBT reagent (200 mM carbonate buffer, pH 10.3, containing 500 μM NBT and 2% Triton X-100) was added and incubated for 30 min at 37 °C. The absorbance of the sample was measured at 530 nm on a spectrophotometer. The dihydroxyacetone, between 0 and 1000 μM, was used as a standard to draw the calibration curve.

For measuring g-LDL,[33] LDL was isolated by using the heparin precipitation method (Gavella, Lipovac, Car, & Vucic, 2002) from the rat serum. LDL precipitate was collected by centrifugation, washed twice with 0.2 M $CaCl_2$ and dissolved in 5% NaCl containing 0.5% EDTA (Cornwell & Kruger, 1961). After that, 0.9 M oxalic acid (v:v 1:2) was added and the samples were incubated at 85 °C for 20 h. After cooling, proteins were precipitated by adding TCA (up to 10%) and the supernatants were assayed for glycated products. In this method, after addition of oxalic acid and boiling, glucose is dehydrated and 5-HMF is produced as a chromogen by adding thiobarbituric acid. The standard curve was plotted using 5-HMF (Fluckiger & Gallop, 1984).

GO and MG were measured by HPLC (Deng & Peter, 1999). The HPLC system consisted of a KNAUER, Smartline Manager 5000 manual, Smartline pump 1000, a Smartline UV detector 2000 (KNAUER Corporation, Germany) and an analytical column, KNAUER C18 (250 mm 4.6 mm; 5 μ). The DNPH derivatives of GO and MG calibrators were used for chromatography assay. The elution was isocratic with 20 mM phosphate buffer, pH 4.6, containing 32% acetonitrile and 8% of 2-methylpropanol at a flow rate of 1.0 ml/min. The DNPH derivatives were analyzed via a UV-detector at 330 nm.

PEN was measured by the modified method of Słowik-Żłka et al. (2004) in order to undertake the experiment in a shorter run time. The HPLC system consisted of a KNAUER HPLC system with an Rf-10AXL Shimadzu fluorescent detector and the same column as above. The chromatography assay was briefly undertaken as follows: 100 μl of the sample was lyophilized and then hydrolyzed by 50 μl of 6 N HCl at 110 °C under nitrogen atmosphere for 16 h, and subsequently neutralized with 100 μl of NaOH (5 M) and 200 μl of 500 mM of phosphate buffer (pH 7.4), and then filtered through a 0.45 μm Millipore filter. After that, it was diluted 20-fold with PBS. Filtered samples (50 μl) were then injected into a C-18 reverse-phase analytical column using an online fluorescence detector at excitation/emission wavelengths of 335/372 nm. A linear gradient of three eluents (A, B and C) was used. Eluent A was 10 mM of HFBA[34] in water and eluent B was 25% acetonitrile + 75% H_2O + 10 mM of HFBA; and eluent C was acetonitrile. The elution profile was as follows: 0 to 7 min, 60% A and 40% B; 7 to 11 min, 100% C; 11.5 to 15 min, 60% A and 40% B. The flow rate was maintained at 1.2 ml/min through the chromatographic run.

The AGEs were determined according to the method of Kalousova, Skrha, & Zima (2002). The sample was diluted 1:50 with phosphate buffer (pH 7.4) and fluorescence intensity was recorded at the emission maximum (440 nm) upon excitation at 370 nm using the spectrofluorometer Shimadzu, Model RF-5000 (Shimadzu, Kyoto, Japan). Fluorescence intensity[35] was expressed as arbitrary units.[36]

Determination of the effectiveness of treatments on glycation inhibition

The percentage of inhibition of the glycated product was calculated according to Eq. (2) (Beisswenger, Moore, Brinck-Johnsen, & Curphey, 1993), by using the data of Table 1 for g-Alb, GO, MG, PEN, AGEs in the presence or absence of Cys.

$$\text{Glycation inhibition}(\%) = 100 \times \left[1 - \frac{(\text{DT} - \text{N})}{(\text{D} - \text{N})}\right] \quad (2)$$

where N, D and DT were the values of the desired product in the normal, diabetic–atherosclerotic and diabetic–atherosclerotic rats under treatment, respectively.

Determination of LDL glycation and oxidation products

For estimation of LDL oxidation by the baseline level of diene conjugation[37] in LDL lipids, the following procedure was used. At first the lipids were extracted from LDL samples (100 ml) by chloroform–methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm. Absorbance units were converted to molar units using the molar extinction coefficient of $2.95 \times 10^4\ M^{-1}$ (Ahotupa et al., 1998). For measuring the OFP[38] of LDL, dried residues were diluted 20 times with phosphate buffer 100 mM, pH 7.4; and the emission was measured at 430 nm with excitation at 360 nm (Esterbauer, Gebicki, Puhl, & Jürgens, 1992).

Determination of HS CRP

Hs CRP[39] was measured with CUSABIO ELISA kit (Wuhan, Hubei, China).

Determination of protein in the urine

Protein in the urine was measured with turbidimetric method (Shahangian, Brown, & Ash, 1984). Briefly, 100 μl of TCA 3% was added to 1 ml of distilled water, sample and protein calibrator, respectively. Absorption of sample after 5 min was compared with protein standards at 420 nm.

Pathological study

At the end of the experiment, the animals were killed and their whole aortas were collected and any adhering tissue was removed. All specimens were fixed in 10% buffered formalin, processed and paraffin embedded. Transverse and longitudinal sections of each segment were submitted on one cassette. Then using H&E the sections were stained and sent for pathological examination to a pathologist.

The in vitro experiments

Purification of RSA and its incubation in different situation

Rat serum albumin[40] was extracted using TCA and ethanol, by the modified method of Ohkawara et al. (2002).

Then, three series of the duplicated tubes containing, Alb (10 mg/ml of RSA), Alb + Glc, (10 mg/ml RSA + 50 mM Glc) and Alb + Glc + Cys (same concentrations of RSA and Glc + 0.05% Cys) were incubated for 3 months at 37 °C. Each solution was prepared in 100 mM sodium phosphate buffer containing sodium azide (0.1 mM), to prevent degradation (Vinson & Howard, 1996) and inhibit bacterial growth (Dearlove, Greenspan, Hartle, Swanson, & Hargrove, 2008), and EDTA (1 mM) to prevent artificial oxidation (Brown, Mahroof, Cook, Van Reyk, & Davies, 2006). The reaction mixtures were then sterilized by filtration (0.45 μm pore filter). The aliquots of samples were prepared after 21 days for measuring g-Alb, GO and MG; and after 90 days for determination of AGEs and PEN. All aliquots were maintained at −70 °C until

[32] Fructosamine.
[33] Glycated LDL.
[34] Heptafluorobutyric acid.
[35] FI.
[36] AU.
[37] DC.
[38] Oxidation fluorescent product.
[39] High sensitivity C-reactive protein.
[40] RSA.

Table 1
The effect of Cys on FBS, insulin, HOMA-IR, glycation products (FA, g-Alb, g-LDL, GO, MG, PEN and AGEs), and Cr in the serum and protein in the urine (PU) of the normal and diabetic rats with or without receiving Cys.

Row	Parameter	Unit	Groups			
			N	NT	D	DT
1	FBS	mM	4.48 ± 0.25[b]	5.04 ± 0.20[b]	15.55 ± 0.46[a]	9.45 ± 0.33[a,b]
2	FA	μM	150.00 ± 8.80[b]	126.00 ± 7.60[a,b]	626.00 ± 41.20[a]	477.00 ± 28.30[a,b]
3	g-Alb		80.00 ± 4.60[b]	67.70 ± 4.40[a,b]	402.00 ± 20.70[a]	316.00 ± 14.40[a,b]
4	g-LDL		51.13 ± 1.70[b]	38.06 ± 0.85[a,b]	185.90 ± 6.77[a]	144.50 ± 7.48[a,b]
5	GO		15.60 ± 0.80[b]	13.70 ± 0.70[b]	103.80 ± 6.00[a]	46.20 ± 3.00[a,b]
6	MG		16.80 ± 0.95[b]	11.90 ± 0.80[b]	93.00 ± 5.10[a]	43.30 ± 3.30[a,b]
7	PEN		42.00 ± 2.73[b]	37.70 ± 6.10[b]	446.00 ± 20.70[a]	348 ± 17.00[a,b]
8	AGEs	FI, A.U	47.66 ± 2.90[b]	39.70 ± 4.80[b]	363.70 ± 19.00[a]	148.3 ± 6.60[a,b]
9	Insulin	μU/ml	17.49 ± 1.36[b]	17.66 ± 1.45[b]	8.98 ± 0.60[a]	11.51 ± 1.04[a,b]
10	HOMA-IR		3.78 ± 0.18[b]	3.55 ± 0.15[b]	6.2 ± 0.35[a]	4.84 ± 0.27[a,b]
11	Cr	μM	61.50 ± 4.24[b]	57.70 ± 4.09[b]	96.50 ± 5.74[a]	74.25 ± 4.32[a,b]
12	PU	mg/24 h	13.00 ± 0.90[b]	9.41 ± 0.80[b]	347.50 ± 17.50[a]	115.58 ± 5.94[a,b]

[a] Indicates significance of data comparing group N with other groups ($P < 0.001$).
[b] Indicates significance of data comparing group D with other groups ($P < 0.001$).

they were used for analysis. All measurements were done at least three times and if there was uncompatibility, the experiment was repeated. The g-Alb, MG, GO, PEN and AGEs in the reaction mixtures were measured using the methods explained in Section 2.3.2.

Purification of LDL and determination of its glycation and oxidation products

LDL was isolated with heparine from the serum of normal rats and incubated in the same conditions as explained in Section 2.3.1, for one week. The samples were then separated for measurement of g-LDL and oxidated LDL using the methods explained in Section 2.2.7. In addition, the oxidative modification of both native and glycated LDL by $CuSO_4$ in the presence or absence of Cys was compared photometrically, by measuring the lag phase of conjugated diene formation at 234 nm (Esterbauer, Striegl, Puhl, & Rotheneder, 1989).

Determination of the effectiveness of treatments on glycation inhibition

The percentage of inhibition of the glycated product in the in vitro condition was calculated according to Eq. (2) and the data presented in Table 5 for RSA (N), RSA + Glc (D) and RSA + Glc + Cys (DT).

Statistical analysis

All data were expressed as mean ± S.D. Statistical comparisons were performed by Tukey HSD multiple analysis of variance (MANOVA) test. The correlation between PU and other clinical variables was determined by multiple regression analysis using SPSS version 16 Statistical, significance was defined as $P < 0.05$.

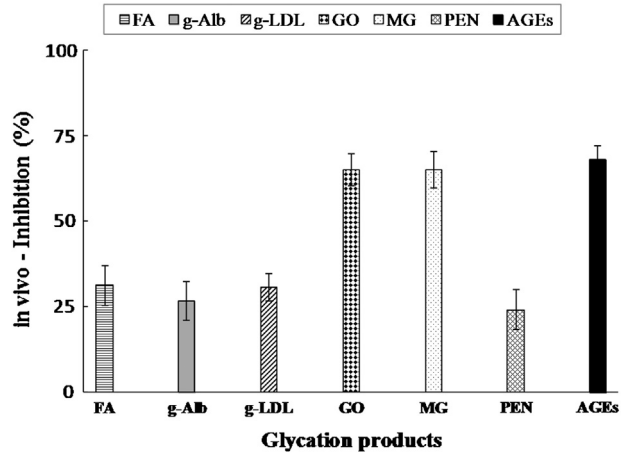

Fig. 1. The percentage of the inhibitory effect of Cys on the formation of the early, intermediates and end products of glycation in the diabetic–atherosclerotic rats treated with Cys.

Results

The results of the in vivo experiment

Table 1 represents the levels of FBS, insulin, HOMA-IR, various glycation products and Cr in the sera and PU of all groups of rats. All of the named parameters were significantly ($P < 0.001$) increased in the diabetic group in comparison with the normal group, except insulin secretion which decreased due to diabetes induction. Cys could, at least partially, compensate for ($P < 0.001$) these changes in diabetic rats. Comparing the data of normal groups with or without Cys treatment showed that except in the FA, g-Alb and g-LDL, there were no significant changes in the other parameters. This indicates the beneficial effect of Cys treatment, even in the normal rats.

As indicated in Table 1, the inhibitory effect of Cys on all of the early, intermediates and end products of glycation (FA, g-Alb, g-LDL, MG, GO, PEN and AGE) in the serum of diabetic rats was not the same. Thus, the percentage of inhibition of each of them was calculated using the data in Table 1 and Eq. (2), and is shown in Fig. 1.

Cys therapy significantly ($P < 0.001$) reduced the increased level of serum Cr in diabetic rats. It also significantly ($P < 0.001$) decreased the protein excretion in the urine of diabetic rats.

The effect of Cys on lipid profile is shown in Table 2. The normal and diabetic groups under the treatment with Cys showed significantly ($P < 0.001$) lower levels of TG, Chol and LDL in comparison with the diabetic group without treatment, but their HDL level was significantly higher.

Comparison of the effect of the treatment on oxidation markers including DC, FOP and AOPP, as well as the inflammatory marker, Hs CRP, is shown in Table 3. The activities of GLO-I and GLO-II in all of the

Table 2
The effect of L-Cys on lipid profile in normal and diabetic rats without (N & D, respectively) or with Cys treatment (NT & DT, respectively).

Row	Group name	TG	Chol	HDL	LDL
		mM			
1	N	0.93 ± 0.02[b]	2.22 ± 0.07[b]	1.40 ± 0.05[b]	0.039 ± 0.01[b]
2	NT	0.75 ± 0.03[a,b]	1.79 ± 0.05[a,b]	1.08 ± 0.03 [a,b]	0.35 ± 0.01[b]
3	D	2.83 ± 0.12[a]	6.46 ± 0.24[a]	0.43 ± 0.01[a]	4.75 ± 0.24[a]
4	DT	1.47 ± 0.06[a,b]	5.63 ± 0.11[a,b]	0.61 ± 0.02[a,b]	4.35 ± 0.20[a,b]

[a] Indicates significance of data comparing group N with other groups ($P < 0.001$).
[b] Indicates significance of data comparing group D with other groups ($P < 0.001$).

Table 3
Comparison between DC, FOP, AOPP and Hs CRP in different groups of rats. N, normal; D, diabetic–atherosclerotic; NT and DT, normal and diabetic groups received Cys, respectively.

Group	DC (μM)	FOP (AU)	AOPP (μM)	Hs CRP (ng/ml)
235 ± 13.07[b]	28.85 ± 0.99[b]	248.80 ± 12.11[b]	15.98 ± 0.90[b]	N
232 ± 12.99[b]	19.65 ± 1.06[a,b]	245.33 ± 13.71[b]	12.95 ± 0.66[b]	NT
596.13 ± 32.66[a]	89.1 ± 3.25[a]	514.66 ± 22.37[a]	108.33 ± 8.40[a]	D
554.50 ± 29.63[a,b]	64.27 ± 3.89[a,b]	487.11 ± 18.92[a,b]	67.61 ± 5.67[a,b]	DT

[a] Indicates significance of data comparing group N with other groups ($P < 0.001$).
[b] Indicates significance of data comparing group D with other groups ($P < 0.001$).

mentioned groups are shown in Fig. 2a and b. Induction of diabetes increased all of the oxidation products; however, the levels of DC, OFP, AOPP and Hs CRP decreased significantly ($P < 0001$) in the diabetic group treated with Cys. Furthermore, the decreased glyoxalase activity system (both GLO-I and GLO-II) in diabetic group (D) was significantly ($P < 0001$), but not completely, compensated for in the diabetic group under treatment (DT).

The correlation between PU and other measurable parameters are presented in Table 4 All parameters, except HDL, insulin and GLO system activity, showed a high positive correlation with PU. The level of the multiple correlation coefficient with PU ($R = 0.892$) indicates a high level of prediction. These parameters significantly predict PU ($F = 556.87$ (11, 12), $P < 0.005$).

The effect of Cys on atheromatous plaque formation in diabetic–atherosclerotic rats in comparison with the normal group is shown in Fig. 3a and b. As the results show, the atheromatous plaque was only formed in the diabetic–atherosclerotic rats.

The results of the in vitro study

The inhibitory effect of Cys on the formation of various glycation products of RSA in the presence of Glc, is presented in Table 5. Since

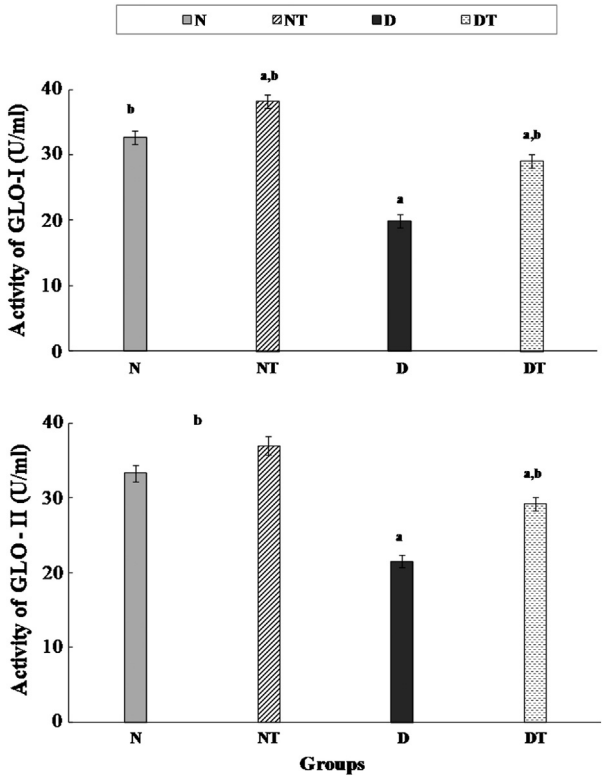

Fig. 2. The activities of the glyoxalase I (a), and glyoxalase II (b) in different groups. (a) Indicates significance of data comparing group N with other groups ($P < 0.001$). (b) Indicates significance of data comparing group D with other groups ($P < 0.001$).

Table 4
Correlation between proteinuria (PU) with Glc, insulin, HOMA-IR, glycation products (g-Alb, GO, MG, PEN and AGEs), lipid profile (TG, Chol, HDL and LDL), oxidation markers (DC, OFP and AOPP), Cr, glyoxalase system (GLO-I and GLO-II) and Hs CRP in the diabetic–atherosclerotic rats treated with Cys.

Parameter	r	P value <
Glc	0.993	**0.001**
Insulin	−0.923	
HOMA-IR	0.955	
FA	0.961	
g-Alb	0.947	
g-LDL	0.878	
GO	0.987	
MG	0.989	
PEN	0.943	
AGEs	0.993	
TG	0.987	
Chol	0.925	
HDL	−0.902	
LDL	0.900	
Cr	0.948	
DC	0.987	
OFP	0.899	
AOPP	0.969	
GLO-I	−0.940	
GLO-II	−0.967	
Hs CRP	0.905	

this inhibitory effect was not similar on all of the named products, the percent of inhibition of each of the g-Alb, MGO, GO, PEN and AGE in the in vitro condition was calculated using Eq. (2) and shown in Fig. 4.

Fig. 5 compares the contribution of each of the Maillard reaction products to a total (100%) inhibition of glycation by Cys in both in vivo and in vitro conditions.

The effect of Cys on the inhibition of the formation of glycation and oxidation products of LDL is presented in Table 6. As the results indicated in the presence of Glc, concentrations of all of the glycated, diene conjugation and fluorescent oxidation products of LDL were increased, but in the presence of Cys, their production was much less.

Discussion

In this study the beneficial effects of three month Cys treatment on preventing the atheromatous plaque formation and lowering the risk of diabetic complications, including cardiovascular and renal risk factors, are shown. Cys therapy significantly reduced hyperglycemia, and the concentration of various glycated and oxidation products, improved the lipid profile and inflammatory marker, decreased Cr and PU, and increased glyoxalase system activity in the diabetic rats fed atherogenic diet. In addition, the inhibitory effect of this treatment on the in vitro glycation of RSA and LDL was demonstrated; a significant correlation was also observed between the in vivo and in vitro results.

The early, intermediates (Lu et al., 2011) and end products (Sourris & Forbes, 2009) of the Maillard reaction have a significant role in the development of nephropathy and accelerated atherosclerosis in diabetes. Thus, using strategies to prevent the formation of these products, in addition to controlling blood Glc, could reduce the risk of the diabetes complications. As indicated in the previous studies, the results obtained here indicate that after diabetes induction, the atherogenic diet seriously increased the risk of both ND and CVD in rats.

The beneficial role of sulfur containing amino acids in the control of hyperglycemia has been considered in a recently published review paper (Manna, Das, & Sil, 2013). Here we showed that treatment of the diabetic–atherosclerotic rats with Cys reduced FBS. The hypoglycemic effect of this amino acid has also been previously reported in ZDF[41] rats (Jain, Velusamy, Croad, Rains, & Bull, 2009) and in diabetic rats under

[41] Zucker diabetic fatty.

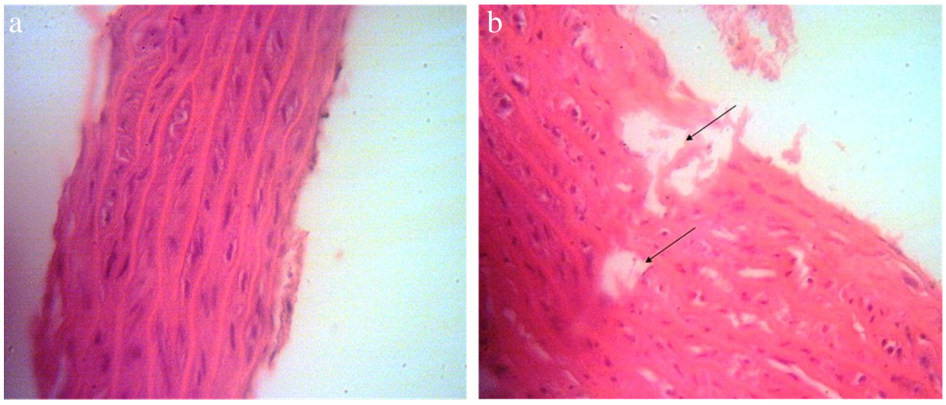

Fig. 3. The effect of the desired treatments on atheromatous plaque formation in normal and diabetic–atherosclerotic rats (stained by H&E; original magnification ×200). (a) Normal aorta in diabetic group treated with Cys and normal group. (b) Atheromatous plaque formation in untreated diabetic group.

treatment with both Cys and metformin (Salman, Refaat, Selima, El Sarha, & Ismail, 2013). In contrast, some published data indicated that Cys (200 mg/Kg) therapy by gavage, alone or in combination with taurine, had no effect on blood glucose level of diabetic rats, even after 8 weeks (Tappia, Xu, Rodriguez-Leyva, Aroutiounova, & Dhalla, 2013) The hypoglycemic effect of other amino acids like Lys (Jafarnejad et al., 2008) and Gly (Bahmani et al., 2012) in the diabetic rats has also been reported by us. However, the present results show that lower concentrations of Cys, than the mentioned amino acids, are needed to decrease the FBS.

The beneficial effect of Cys on insulin sensitivity of diabetic rats shown here, confirms previous reports (Jain et al., 2009; Salman et al., 2013). Based on the results of the calculated HOMA-IR, it can be concluded that the mechanism of hypoglycemic activity of Cys is possibly through improving the pancreatic β-cells, which is consistent with the antioxidant property of Cys, and decreasing of insulin resistance. The observed decrease in Hs CRP level, after Cys administration, that was increased due to diabetes induction is the other reason for beneficial effect of this treatment on β-cell function and decreasing the inflammatory response as a consequence of oxidative processes.

It has been previously reported that Cys treatment inhibited the glycation of hemoglobin in ZDF (Jain et al., 2009). Hemoglobin is a cytosolic protein extensively found in RBC. In the present study, we investigated the effect of Cys in an extracellular protein, albumin, which is involved in many biological processes, including osmotic pressure, transfer of different molecules (free fatty acids, drugs, toxins, etc.) in the blood stream and binding to various cations. In addition, we investigated the inhibitory effect of Cys on another biomacromolecule (LDL) involved in the CVD. Our results indicated that this sulfur containing amino acid inhibited the formation of various glycated products (MG, GO, PEN and AGEs) of RSA and LDL, with various degrees, in both test tubes (in vitro), and in rat (in vivo). These results also indicated that the levels of FA, g-Alb and g-LDL in the normal rats received Cys were even lower than the untreated group. Therefore, Cys therapy could be useful to significantly decrease the important risk factors of diabetes complications.

The inhibitory activity of S-allyl cysteine on Amadori product of bovine serum albumin and in vitro AGEs formation (Ahmad, Pischetsrieder, & Ahmed, 2007), the potential of Cys and cysteamine to trap MG (Vidal et al., 2014), and the in vitro inhibitory effect of Cys on the early LDL oxidation (DC) have been reported (Patterson, Lamb, & Leake, 2003). All of these reports are consistent with the observed results here.

However, as indicated in Fig. 5, the inhibitory effect of Cys on RSA glycation products is slightly different in two situations, in vitro and in vivo. So that, inhibition of the in vitro formation of g-Alb and PEN was more than that of the in vivo. In contrast, the anti-glycating activity of Cys against the other glycated products was stronger in the in vivo than that in vitro. To find the cause of these differences, we determined the activities of the glyoxalase system, as a potent system involves in the detoxification of MG and other reactive aldehydes. The activities of both GLO-I and GLO-II were significantly decreased due to diabetes induction. As the data shows, Cys treatment significantly ($P < 0.001$) increased glyoxalase system activity, in the in vivo. There are at least two possibilities for these observations. First, these enzymes were glycated in the hyperglycemic condition and because of the conformational changes, their activities have decreased. But in the presence of a glycation inhibitor and an antioxidant, like Cys, their enzymatic activities were restored. Similar phenomena have been previously shown in various enzymes and proteins in the serum, plasma, extracellular matrix, cytosol, and even in the nucleus by us (Bathaie, Jafarnejad, Hosseinkhani, & Nakhjavani, 2010; Bathaie, Nobakht, Mirmiranpour, Jafarnejad, & Moosavi-Nejad, 2011; Jafarnejad et al., 2008; Mirmiranpour, Bathaie, Khaghani, Nakhjavani, & Kebriaeezadeh, 2012; Rahmanpour & Bathaie, 2011). Second, Cys administration raised the GSH concentration (Griffith, 1999), which in order, by increasing the substrate concentration, the activity of the glyoxalase system and thus detoxification of dicarbonyl compounds have increased. Both of these mechanisms should be extensively investigated in future studies.

To continue, we investigated the effect of Cys on various parameters involved in consequences of diabetes. The improvement effect of Cys therapy on TG, Chol and HDL of both normal and diabetic rats was

Table 5
The effect of Cys on the in vitro formation of various glycated products of rat serum albumin (RSA).

Row	Treatment	g-Alb	GO	MG	PEN	AGEs
		μM				(AU)
1	RSA	120.00 ± 3.40	9.45 ± 0.10	3.85 ± 0.10	4.60 ± 0.15	55.00 ± 1.00
2	RSA + Glc	1150.00 ± 10.10	30.45 ± 0.40	9.85 ± 0.20	21.80 ± 0.11	499.00 ± 5.10
3	RSA + Glc + Cys	381.00 ± 4.20	24.10 ± 0.47	6.18 ± 0.16	12.50 ± 0.23	268.00 ± 4.20

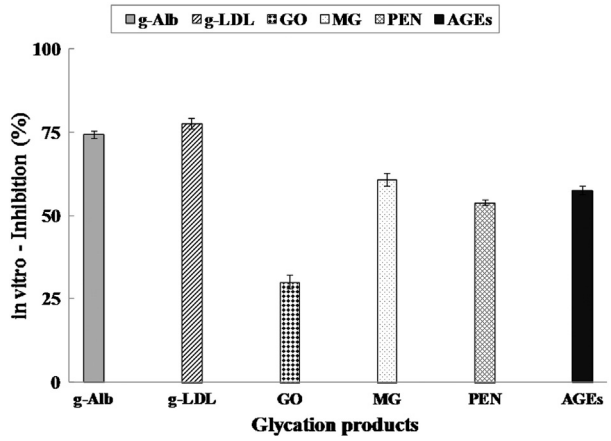

Fig. 4. The percentage of the inhibitory effect of Cys on diverse glycation products of rat serum albumin (RSA) in the in vitro condition.

observed, but LDL only decreased in the diabetic group treated with Cys ($P < 0.001$). As indicated in the results, Cys not only reduced LDL level in diabetic–atherosclerotic rats, but also inhibited the LDL glycation and oxidation, thus it exerts a strong anti-atherogenic activity, which is confirmed by other results.

The inhibitory effect of Cys on protein oxidation (AOPP formation) which has been previously reported in the ZDF (Jain et al., 2009), is also shown in the present study using Wistar albino rat. This data accompanying with the Cys inhibitory effect on the formation of DC and FOP (markers of early- and end-LDL oxidation), and inhibition of various AGE products, confirm the role of Cys as a strong inhibitor of ROS formation.

Serum Cr was significantly ($P < 0.05$) increased due to the diabetes induction; however, it was significantly reduced in this group due to the treatment with Cys. The protein concentration in the urine of the diabetic–atherosclerotic rats was also significantly ($P < 0.005$) increased; and it was decreased due to the Cys therapy, but both of these parameters were still higher than the normal levels. These results indicated that diabetes induction accompanied with the atherogenic diet cause the induction of DN in the rats, however Cys reduced the risk of this complication. A significant decrease in microalbuminuria of diabetic rats treated with Lys (Jafarnejad et al., 2008), and the reduction of PU in diabetic rat treated with L-arginine has been reported previously (Reyes, Karl, Kissane, & Klahr, 1993), but Cys was stronger than the named amino acids and exerts its role at lower concentration.

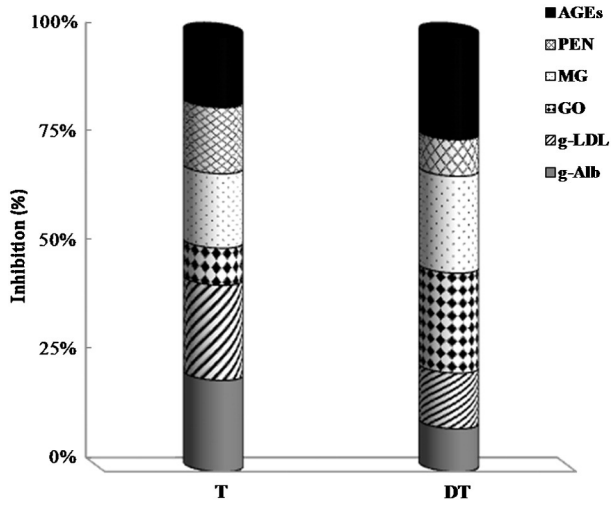

Fig. 5. Contribution of each of the Maillard reaction products to a total (100%) inhibition of glycation by Cys in both in vitro and in vivo studies.

Table 6
The effect of Cys on the in vitro formation of glycated and oxidized products of LDL.

Row	Treatment	g-LDL	DC	FOP
		μM	AU	
1	LDL	19.50 ± 0.70	16.94 ± 0.57	15.17 ± 0.44
2	LDL + Glc	328.00 ± 7.07	135.56 ± 4.55	500.01 ± 10.84
3	LDL + Glc + Cys	87.00 ± 1.41	36.60 ± 1.18	46.12 ± 1.36

PU has been known as an important predictor of DN (Marso et al., 1999) and CVD (Mitch et al., 2004). The correlation between PU with AGEs and Chol has also been reported (Nakamura et al., 2010). Here, the correlation between PU and each of the Glc, glycation and oxidation products, lipid profiles, Hs CRP, GLO system activity and Cr was investigated. As shown in Table 3, the risk of diabetic nephropathy (based on the level of correlation) rises with the reduction of HDL, insulin and GLO system activity and the increase of the other mentioned parameters. Therefore, it is suggested that in addition to FBS, the levels of the glycation and oxidation products could also be the predictors of the risk of DN and CVD. As indicated in the present study, Cys as a multifunctional compound with hypoglycemic, antioxidant and anti-inflammatory activities, inhibited protein glycation, could trap the dicarbonyl compounds (Mehta, Wong, & O'Brien, 2009) and induced the glyoxalase system activity, which in order reduced more the level of glycoxidation compounds. Therefore, it should be administered to the diabetic patients to reduce the chance of diabetic complications.

In conclusion, the results of the present study indicated that Cys as a reducing compound and a glycation inhibitor decreases all of the risk factors of DN and CVD in diabetic–atherosclerotic rats, after the three month treatment. In addition, diverse glycation and oxidation products, especially GO, MG and AGEs can be determined and used as the predictors of diabetic complications.

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